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Spring 2019

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# Assessment of Sex-Related Differences of the Osteocyte Lacunar-Canalicular Network Across the Human Lifespan Using Synchrotron micro-Computed Tomography

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**Honors Research Project**

Submitted to

*The Honors College*

**Abstract**

Osteocytes, the most ubiquitous bone cell type, are responsible for bone maintenance and communication as well as mechanotransduction. Osteocytes reside in spaces within the bone matrix called lacunae, which are used as a proxy for the cells themselves in X-ray imaging. Previous studies have revealed that lacunar volume was reduced in females with increasing age, thus likely contributing to bone frailty and diseases such as osteoporosis. The implications of diseases caused by changes in the bone osteocyte lacunar-canalicular network are not yet fully understood. Since much of the past research revolved around gross bone morphology, this study investigated age and sex-related differences in the bone cellular network of the human species. Utilizing synchrotron radiation-based micro-Computed Tomography, male (n=12) and female (n=8) cortical bone specimen from the mid-diaphyses of the left femora were assessed for tissue volume ($\mu m^3$), canal volume ($\mu m^3$), canal surface ($\mu m^2$), cortical porosity (%), canal surface to tissue volume ($\mu m^{-1}$), canal diameter (μm), canal separation (μm), number of canals, and number of lacunae. These parameters were compared between age, sex, and the interaction between both factors. In regard to number of lacunae and their density, a statistically significant reduction was observed with age ($p = 0.017$) but not with sex or the interaction variable. Thus, lacunar density was reduced with age, but no significant differences between males and females were observed. Limitation in sample size prevented a more extensive result. As such, further investigation is encouraged to confirm the reduction of lacunar volume over the human lifespan.

## 1. Introduction

Human bone is living tissue; therefore, it is always adapting to biomechanical stressors, hormones, diet, and disease processes. Bone consists of two linked but distinct types: cancellous and cortical. Cancellous bone is extremely porous and spongy and is the inner lattice of the bone. Cortical, on the other hand, is the outer shell which surrounds the cancellous bone[1]. Cortical bone is most distinct within the diaphysis of bone, whereas cancellous bone is prominent within the metaphyses and ephiphyses[1]. Bones also consist of a variety of cells which include osteoblasts, osteoclasts, and osteocytes. Osteoblasts synthesize bone and form new bone matrices primarily composed of collagen, which then mineralize to become bone. Hydroxyapatite, an inorganic mineral also produced by osteoblasts, is sophisticatedly organized within the collagen matrices, thus aiding the formation and solidification of the bone's structure[2]. Osteoclasts are responsible for bone remodeling and resorption[3]. These processes of bone resorption and remodeling occur in a balanced manner in order to maintain this delicate homeostatic process of remodelling[4]. Osteocytes are mature bone cells that are involved in bone maintenance and calcium homeostasis[3]. Osteocytes are the most abundant cells found in bone[5] and are the only cell type that are embedded in bone[6]. The three-dimensional (3D) network that osteocytes and their lacunae form are situated inside canaliculi[6]. Together, they are collectively known as the lacunar-canalicular network[6] (LCN). Osteocytes are important when observing mechanotransduction and mechanosensation of bone because the LCN plays a large role in this process.

Since no two human genomes are identical, there is documented variation from individual to individual, with a greater distinction between the sexes in regard to gross bone morphology and nuances in bone micromorphology. Males and females undergo sexually dimorphic biological processes throughout their respective lifetimes. Women, for example,

experience a period in life referred to as menopause when the menstrual cycle ceases to occur. This occurrence is due to the decreased production of ovarian hormones, such as estrogen[7]. Estrogen is a necessary element that protects against bone loss[7]; therefore, when low, it can lead to increased skeletal resorption[7]. Beyond the apparent gross morphological adjustments that the body experiences, the bones' vast cellular network is also modified. A shift in osteocyte lacunar volume in the cortical bone of females occurs after this midlife event. The LCN has been observed to be reduced by an average of 40% when comparing females in their twenties versus those in their seventies[8]. Due to this phenomenon, women have a greater risk of suffering an osteoporotic fracture during their lifetime. Bone frailty has been attributed to the reduction of canaliculi rather than the decrease of lacunar density[8]. The presented decline of female bone density with increasing age has led to further studies in order to gain additional information regarding age-related bone degradation. Osteoporosis has posed a prominent problem for a large portion of the population, especially in females. According to the National Osteoporosis Foundation, 54 million Americans suffer from osteoporosis and low density which places them at a high risk to develop the disease later in life.

In the present study, we investigated the age and sex-related differences in the human bone cellular network. We hypothesized that the men and women of advancing age will have reduced lacunar density compared to their younger counterparts, with women having the largest reduction of canaliculi due to estrogen deficiency. In order for in depth analysis to occur, high resolution scans of each sample were taken using synchrotron radiation-based micro-Computed Tomography (SRμCT). The purpose of this study was to allow for a more thorough scientific understanding on lacuna-canicular density across the human lifespan with regard to sex and age.

## 2. Methods and Materials

### *2.1 Procuring the Samples*

Cortical bone samples examined in this study were from the mid-diaphyses of left femora from American males and females aged between 20 and 90 years. Many of the specimens were obtained from cadavers donated to local medical schools as well as other medical institutions, which include the Office of Chief Medical Examiner of the City of New York, Northeast Ohio Medical University, and the University of Toledo. They were removed from the body using an oscillating saw and were 5-7 cm in length. In this study, eight female samples and twelve male samples were analyzed. One of the eight females and five of the twelve males were under 45 years old.

*2.2 Cleaning the Samples*

Prior to sectioning, the bone specimens were cleaned of all soft tissues. This cleaning was executed by soaking the bone in a solution of Tergazyme Detergent (Sigma-Aldrich, St. Louis, MO) and water for 3-6 hours, whilst being heated in a crockpot or incubator. After the soft tissue softened, it was manually scraped off the bone sample using dental tools. The bones were then fixed in 70% ethanol for at least 24 hours before being left out to dry for proper sectioning.

*2.3 Sectioning and Coring the Samples*

Using a Buehler IsoMet 1000 precision saw, 5-mm sections were obtained using a diamond-tipped wafer saw blade. A mixture of IsoCut fluid and water was used to lubricate the blade. The bone sample was epoxy-glued onto a slide, which was then mounted onto a chuck. The chuck fit onto the saw and 5-mm sections were sliced. To ensure straight, clean slices, the bone specimens were lined up completely perpendicular to the diamond blade before being slowly brought down on the blade. The entire sectioning process was monitored to identify and correct any problems as early as possible.

Cores from the center of the anterior portion of the femora were required for imaging. After the 5-mm sections were epoxy-glued onto a solid surface, they were properly mounted and prepared to ensure the plate would not move for the duration of the drilling process. A mill-drill press was then utilized to carefully core and obtain the desired size of 2 x 2 x 5 mm.

*2.4 BioMedical Imaging and Therapy Beamline (BMIT)*

In June of 2018, nine shifts were scheduled at the Canadian Light Source national synchrotron facility in Saskatchewan, Canada for the BioMedical Imaging and Therapy (BMIT) beamline[9]. The micro-CT system produces images of high resolution, with the scans being ~0.9 μm and the set-up specified to scan for osteocyte lacunae (**Figure 1)**. The scan protocol for the SRμCT scans were followed as outlined in Andronowski et al. 2017[10].


**Figure 1:** Example of SRμCT single projection from cortical bone with a cylindrical VOI.

*2.5 Reconstruction and Analysis*

The SRμCT images were reconstructed with the image reconstruction program NRecon 1.7.4.2. This software was utilized to rid our scans of artifacts that may impact the reconstruction process and potentially affect the results of the study. All images were reconstructed within a consistent threshold of values between 0-130, with the image inverted to avoid variance between the different specimen. After reconstruction with NRecon, analysis was conducted using CTAnalyser (CTAn) 1.18.4.0. CTAn was utilized to measure quantitative parameters from the 3D dataset via Individual Object Analasys. The data collected from CTAn included the following: tissue volume ($\mu m^3$), canal volume ($\mu m^3$), canal surface ($\mu m^2$), cortical porosity (%), canal surface to tissue volume

($\mu m^{-1}$), canal diameter (μm), canal separation (μm), number of canals, and number of lacunae **(Table 1)**.

*2.6 Statistical Analysis*

All statistical analyses were carried out via Minitab 18.1. A two-way Analysis of Variance (ANOVA) was run with sex as a factor and age as a covariate to establish an analysis of variance and determine statistical significance (where $p < 0.05$ was considered significant). After an assessment of the descriptive statistics, the raw data, due to a small sample size, was not normally distributed across all measurements. Scatter plots for each response variant were created with a best fit line, showing the overall trend for each measurable variant for both males and females across the ages being observed.

**Table 1:** Raw data collected for measured variables

| Sex | Age | Tissue Volume ($\mu m^3$) | Canal Volume ($\mu m^3$) | Canal Surface ($\mu m^2$) | Cortical Porosity (%) | Canal Surface to Tissue Volume ($\mu m^{-1}$) | Canal Diameter (μm) | Canal Separation (μm) | Number of Canals | Number of Lacunae |
|---|---|---|---|---|---|---|---|---|---|---|
| F | 21 | 1,310,600,000 | 26,434,000 | 19,313,000 | 2.0169 | 0.014736 | 5.9566 | 43.279 | 700 | 45,654 |
| F | 57 | 1,314,800,000 | 14,402,000 | 12,412,000 | 1.0954 | 0.00944 | 4.5916 | 48.964 | 746 | 34,779 |
| F | 60 | 1,279,553,073 | 14,437,194 | 11,952,176 | 1.1283 | 0.0093409 | 5.1937 | 47.494 | 5,259 | 40,847 |
| F | 62 | 1,310,590,000 | 9,453,300 | 8,343,400 | 0.72389 | 0.0063889 | 4.7816 | 55.304 | 534 | 28,317 |
| F | 74 | 1,307,728,610 | 11,935,456 | 9,580,601 | 0.912686 | 0.00732614 | 5.1696 | 55.845 | 452 | 26,426 |
| F | 76 | 1,302,105,091 | 8,266,470 | 7,328,564 | 0.6348543 | 0.0056824 | 4.8481 | 8.066 | 745 | 27,893 |
| F | 88 | 1,295,738,047 | 9,816,720 | 8,567,011 | 0.7576161 | 0.00661168 | 4.9093 | 55.154 | 1,018 | 31,410 |
| F | 90 | 1,281,641,125 | 8,945,303 | 7,437,922 | 0.69795696 | 0.00580344 | 5.1927 | 74.481 | 1,851 | 29,583 |
| M | 20 | 1,313,300,000 | 15,797,000 | 13,282,000 | 1.2028 | 0.010113 | 5.0809 | 47.913 | 716 | 39,867 |
| M | 31 | 1,313,200,000 | 19,367,000 | 14,840,000 | 1.4748 | 0.0113 | 5.4328 | 48.283 | 483 | 34,850 |
| M | 38 | 1,311,700,000 | 15,574,000 | 12,201,000 | 1.1873 | 0.009302 | 5.4399 | 52.719 | 499 | 30,228 |
| M | 45 | 1,318,800,000 | 18,272,000 | 14,072,000 | 1.3855 | 0.01067 | 5.5803 | 49.38 | 490 | 34,160 |
| M | 45 | 1,286,200,000 | 20,356,000 | 15,732,000 | 1.5826 | 0.012231 | 5.5816 | 44.271 | 9,808 | 53,954 |
| M | 53 | 1,310,300,000 | 15,825,000 | 12,768,000 | 1.2077 | 0.009744 | 5.1922 | 50.537 | 576 | 36,495 |
| M | 57 | 1,312,900,000 | 14,808,000 | 11,739,000 | 1.1279 | 0.008941 | 5.2648 | 53.347 | 1,020 | 39,101 |
| M | 60 | 1,308,751,323 | 8,281,049 | 7,583,731 | 0.632744 | 0.00579463 | 4.4114 | 57.323 | 498 | 25,740 |
| M | 64 | 1,316,800,000 | 17,848,000 | 13,702,000 | 1.3555 | 0.010406 | 5.4034 | 52.494 | 595 | 33,794 |
| M | 74 | 1,314,289,720 | 15,300,743 | 12,421,932 | 1.164183 | 0.00945144 | 5.2938 | 50.364 | 856 | 37,204 |
| M | 88 | 1,315,551,340 | 12,860,643 | 10,355,577 | 0.97758582 | 0.00787166 | 5.3285 | 55.733 | 934 | 31,052 |
| M | 94 | 1,300,985,189 | 12,134,979 | 10,146,219 | 0.932753 | 0.007799 | 4.9552 | 53.789 | 512 | 29,688 |

## 3. Results

Utilizing the data output generated from CTAn, statistical analyses were conducted on the tissue volume, canal volume, canal surface, cortical porosity, canal surface to tissue volume, canal diameter, canal separation, number of canals, and number of lacunae. When observing the interaction effect between sex and age, statistical significance was revealed for canal volume ($F_{(1,19)}$=7.20, p=0.016) (**Table 2)**, canal surface to tissue volume ($F_{(1,19)}$=7.14, p=0.017) (**Table 2**), cortical porosity ($F_{(1,19)}$=6.88, p=0.018) (**Table 2**), and canal surface ($F_{(1,19)}$=7.58, p=0.014) (**Table 2**). After analyzing the different variables in response to age, significance was found in canal volume ($F_{1,19}$=26.77, p= 0.000) (**Table 2)**, canal surface to tissue volume ($F_{1,19}$=29.68, p= 0.000) (**Table 2)**, cortical porosity ($F_{1,19}$=25.86, p= 0.000) (**Table 2)**, canal surface ($F_{(1,19)}$=31.00, p=0.000) (**Table 2**), and the number of lacunae ($F_{(1,19)}$=7.12, p=0.017) (**Table 2**). Statistically significant variance was also observed when comparing sex. The measurements that were found to be significant included canal volume ($F_{(1,19)}$=5.15, p=0.038) (**Table 2**), canal surface to tissue volume ($F_{(1,19)}$=5.15, p=0.037) (**Table 2**), cortical porosity ($F_{(1,19)}$=4.99, p=0.040 (**Table 2**), and canal surface ($F_{(1,19)}$=5.36, p=0.034) (**Table 2**). The scatterplots exhibited decline for both sexes, with females being steeper (**Figures 2-5**).

**Table 2**: P-values, where p<0.05 is deemed significant, and F-values for measured variables.

| | Canal Volume | Tissue Volume | Canal Surface to Tissue Volume | Cortical Porosity | Canal Surface | Number of Lacunae | Number of Canals | Canal Separation | Canal Diameter |
|---|---|---|---|---|---|---|---|---|---|
| Age | *p*= 0.000<br>F=*26.77* | *p*=0.209<br>F=*1.71* | *p*=0.000<br>F=*29.68* | *p*=0.000<br>F=25.86 | *p*=0.000<br>F=31.00 | *p*=0.017<br>F=7.12 | *p*=0.795<br>F=0.07 | *p*=0.309<br>F=1.10 | *p*=0.071<br>F=3.74 |
| Sex | *p*=0.038<br>F=*5.15* | *p*=0.602<br>F=*0.28* | *p*=0.037<br>F=5.15 | *p*=0.040<br>F=4.99 | *p*=0.034<br>F=5.36 | *p*=0.414<br>F=0.70 | *p*=0.778<br>F=0.08 | *p*=0.596<br>F=0.29 | *p*=0.475<br>F=0.54 |
| Age*Sex | *p*=0.016<br>F=*7.20* | *p*=0.267<br>F=*1.33* | *p*=0.017<br>F=7.14 | *p*=0.018<br>F=6.88 | *p*=0.014<br>F=7.58 | *p*=0.347<br>F=0.94 | *p*=0.745<br>F=0.11 | *p*=0.748<br>F=0.11 | *p*=0.337<br>F=0.98 |

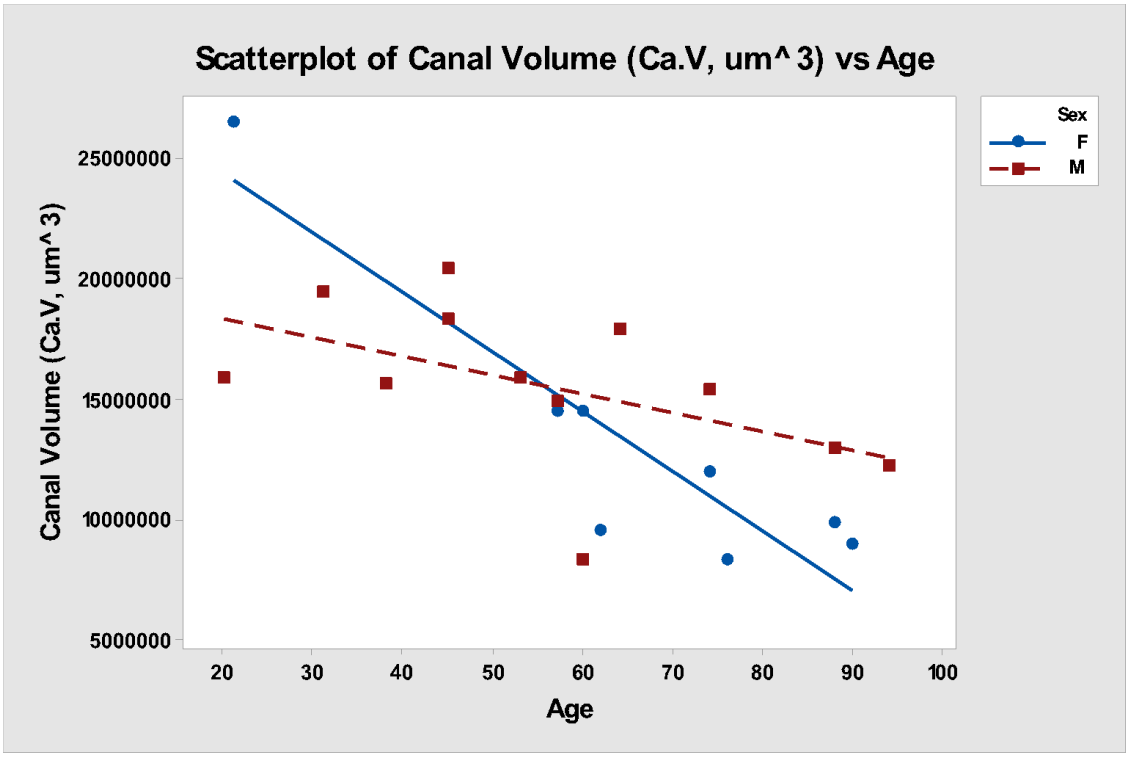


**Figure 2:** Shows male and female canal volume plotted against age as a covariate.

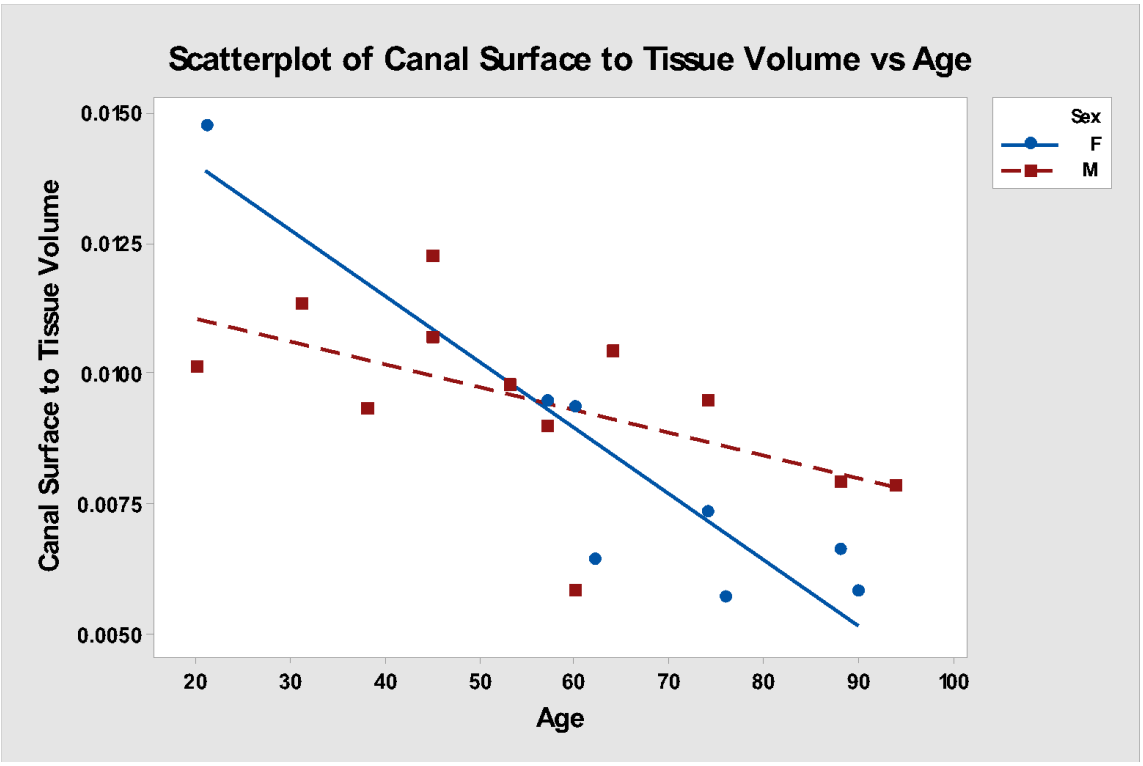


**Figure 3:** Shows male and female canal surface to tissue volume plotted against age as a covariate.

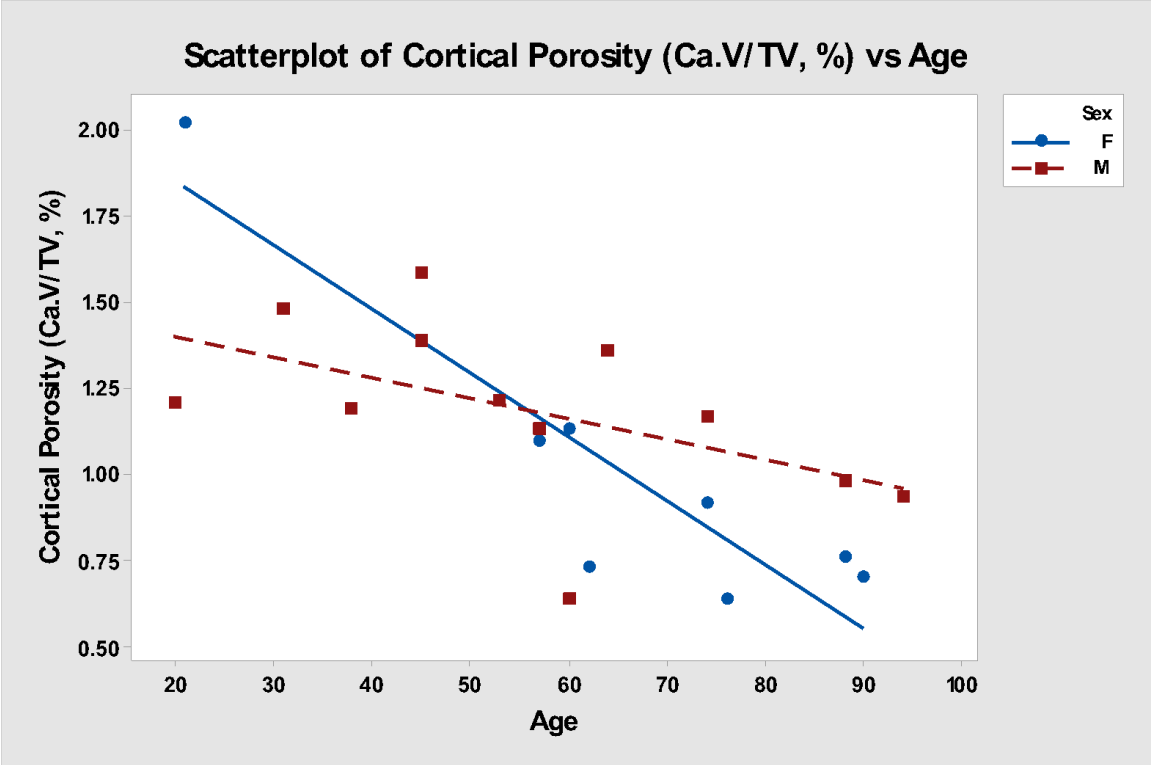


**Figure 4:** Shows male and female cortical porosity plotted against age as a covariate.

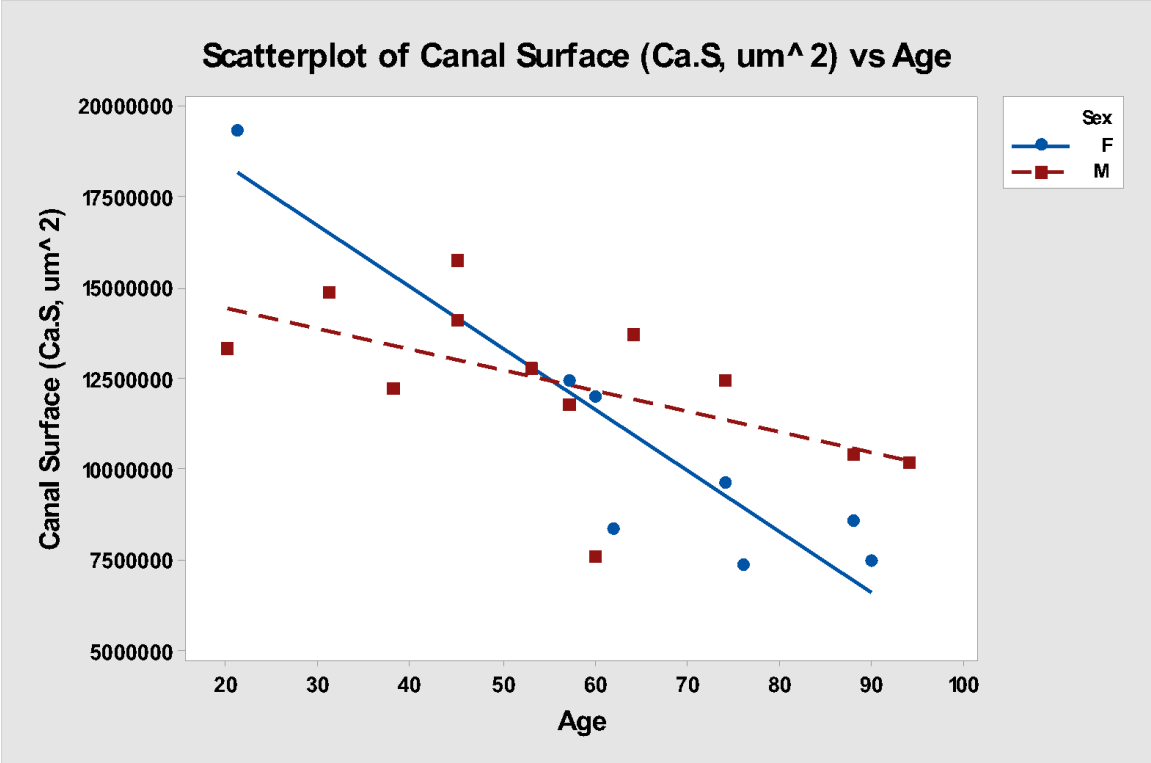


**Figure 5:** Shows male and female canal surface plotted against age as a covariate.

**4. Discussion**

Through the use of SRμCT and subsequent analysis using CTAn, we were able to quantify tissue volume ($\mu m^3$), canal volume ($\mu m^3$), canal surface ($\mu m^2$), cortical porosity (%), canal surface to tissue volume ($\mu m^{-1}$), canal diameter (μm), canal separation (μm), number of canals, and number of lacunae from the femora of males and females across the age spectrum. When discussing tissue volume, the number of canals, and canal separation, we found there to be no statistically significant variance in neither sex nor age. For canal volume, canal surface to tissue volume, cortical porosity, and canal surface, however, significant differences in regard to age, sex, and the interaction between the two were found. Since age is statistically different for these measurements, we can state that as age increases for both males and females, the response variable will change as well. Additionally, it can be noted that women have statistically varying average response variables when compared to men, and when evaluating the interaction between sex and age, we can conclude that age affects these previously listed variables in men and women differently. These results are exhibited in **Figures 2-5** where one can clearly see a steeper, declining slope for females in comparison to males as age increases. The decline represents a reduction of the variable, further supporting the claim that there are indeed significant reductions in the LCN between men and women as age increases. The number of lacunae was found to only be statistically significant with age.

Previous research has been conducted that examined osteocyte lacunar density. Findings by Carter and colleagues concluded that lacunar density declines in women across the human lifespan[11], which is parallel with our findings. Carter and colleagues discovered that the lacunar volume of women over the age of 50 was reduced by a third when compared to their younger counterparts[11]. These findings were also consistent when compared against a study done by Bach-Gansmo et al. which assessed the lacunar volume of male and female iliac crests[12]. They

discovered that although there is no notable significance of densities between males and females, when analyzing both sexes pooled together, a significant reduction was observed with increasing age[12]. Both aforementioned studies utilized SRμCT to image the bone specimens being assessed, thus the methodology is consistent with the current study. With these study comparisons and lack of discrepancies, it can be concluded that there indeed is a reduction of the LCN with age, especially in women.

A limitation of this study is the lack of available specimens for analysis. Obtaining an equal sample size for males and females and an equal distribution of ages within the sexes is extremely difficult. Many older individuals who donate their remains to science have underlying heath disorders or used medications during life that affect bone remodeling and structure. Additionally, younger individuals do not typically die of natural causes; and those who do, typically suffer from drug abuse (which affects bone processes) or do not have their bodies donated to medical schools as they must be autopsied after death. Due to the limitation of available specimens, the data utilized for this study is not normally distributed. A second limitation is the short window of time using the BMIT technology. Being restricted on how many samples were able to be scanned also hindered the success of studying a larger sample size. With only 72 hours at a few hours per scan per year, it was near impossible to scan every specimen during the allotted shifts. This slow, tedious process limited our goal of obtaining a larger sample size.

**5. Conclusions**

Through the utilization of SRμCT, the LCN of human males and females were imaged and subsequently analyzed. This exploration of the osteocyte lacunae in cortical bone proved part of our original hypothesis to be true – women and men both are observed to have a reduction of

the LCN with advancing age. However, the comparison between the sexes was concluded to be non-significant.

**6. Acknowledgements**

Acknowledgement is extended to the Norbert Thompson Research Fellowship Grant for providing the funds which allowed travel to the CLS for data collection. I'd acknowledge our beam team, too, who helped collect these data. I would also like to give special thanks to my sponsor, Dr. Janna Andronowski for helping me conduct my research and allowing me the chance to learn a multitude of techniques, from cleaning and sectioning human bone to analyzing reconstructed scans via various computer software programs. I would also like to thank my readers, Dr. Jordan Renna and Dr. Brian Bagatto, as well as the Honors faculty advisor, Dr. James Holda.

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